

A₁ Adenosine Receptor Modulation of Adenylyl Cyclase of a Deep-living Teleost Fish, *Antimora rostrata*

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Abstract. Low temperatures and high hydrostatic pressures are typical of the deep sea. The effects of these parameters on transmembrane signal transduction were determined through a study of the A₁ adenosine receptor-inhibitory guanine nucleotide binding protein-adenylyl cyclase system in brain membranes of the bathyal teleost fish, *Antimora rostrata* (Moridae). The components of this system were analyzed at 5°C and 1 atm, and the role of the A₁ receptor in the modulation of adenylyl cyclase was determined. The A₁ selective radioligand N⁶-[³H]cyclohexyladenosine bound saturably, reversibly, and with high affinity. The K_d of N⁶-[³H]cyclohexyladenosine estimated from kinetic measurements was 1.11 nM; the K_d determined from equilibrium binding was 4.86 nM. [³²P]ADP-ribosylation of brain membranes by pertussis toxin labeled substrates with apparent molecular masses of 39,000 to 41,000 Da. Basal adenylyl cyclase activity was inhibited in a concentration-dependent manner by the A₁ adenosine receptor agonist N⁶-cyclopentyladenosine (IC₅₀ = 5.08 μM). The inhibition of adenylyl cyclase activity was dependent upon GTP. Basal adenylyl cyclase activity was unaffected by 272 atm of pressure. The efficacy of 100 μM N⁶-cyclopentyladenosine as an inhibitor of adenylyl cyclase was the same at atmospheric pressure and at 272 atm. The inhibition of adenylyl cyclase by the agonist 5'-N-ethylcarboxamidoadenosine (100 μM) at 272 atm was twice that observed at atmospheric pressure. Although consideration

of the effects of low temperature and high hydrostatic pressure on acyl chain order suggest that deep-sea conditions will perturb membrane function, signal transduction by the A₁ receptor system of the bathyal fish *A. rostrata* is not disrupted by deep-sea conditions.

Introduction

The low temperatures and high hydrostatic pressures of the deep ocean may disrupt the biochemical and physiological functions of organisms colonizing this habitat (Siebenaller and Somero, 1978, 1989). Membrane-associated systems are likely to be particularly sensitive because of the ordering effects of these environmental variables on the organization of acyl chains of lipids (Chong and Cossins, 1983; Hochachka and Somero, 1984). Comparisons of homologous cytoplasmic proteins from deep- and shallow-living teleost fishes have established the importance of adaptation to deep-sea temperatures and pressures (Siebenaller and Somero, 1989). Studies of membranes and associated systems in deep-sea organisms indicate that these systems also adapt (*e.g.*, Cossins and Macdonald, 1984, 1986; DeLong and Yayanos, 1985, 1987; Gibbs and Somero, 1989).

To further understand the effects of deep-sea conditions, and to identify potential adaptations of transmembrane signal transduction, we studied the A₁ adenosine receptor and its associated effector elements in brain tissue of a deep-living cold-adapted marine teleost fish, *Antimora rostrata*. The objectives of this study were: (1) to determine whether the A₁ receptor of a typical deep-sea species is coupled to adenylyl cyclase, and (2) to ascertain whether this coupling is functional under the conditions of low temperatures (0–6°C) and high hydrostatic pressures (85–250 atm) experienced by *A. rostrata*. To this end, we undertook a molecular dissection of this

Received 1 September 1989; accepted 30 November 1989.

Abbreviations: ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; [³H]CHA, N⁶-[³H]cyclohexyladenosine; CPA, N⁶-cyclopentyladenosine; G protein, guanine nucleotide binding protein; GTP, guanosine triphosphate; NAD, nicotinamide adenine dinucleotide; NECA, 5'-N-ethylcarboxamidoadenosine; R-PIA, N⁶-phenylisopropyladenosine, R(-) isomer; S-PIA, N⁶-phenylisopropyladenosine, S(+) isomer.

signal transduction system, characterized ligand binding to the A₁ receptor, identified the associated GTP-binding proteins, and determined basal adenylyl cyclase activity and the role of A₁ receptor agonists and GTP-binding proteins in modulation of cAMP accumulation.

A. rostrata is a benthopelagic morid commonly found in the Atlantic and South Pacific Oceans at bathyal depths of 850 to 2500 m (Iwamoto, 1975; Wenner and Musick, 1977). [Pressure increases 1 atm (=101.3 kPa) for every 10 m of depth in the ocean.] *A. rostrata* is replaced by the congener *A. microlepis* in the North Pacific (Small, 1981). Many adaptations to hydrostatic pressure and low temperature have been documented for these species (e.g., Hochachka, 1975; Siebenaller and Somero, 1979; Somero and Siebenaller, 1979; Cossins and Macdonald, 1984, 1986; Avrova, 1984; Yancey and Siebenaller, 1987; Hennessey and Siebenaller, 1987; Gibbs and Somero, 1989).

A₁ adenosine receptor modulation of adenylyl cyclase was selected for two reasons as a model with which to examine pressure and temperature effects on transmembrane signal transduction. First, our previous studies had documented the widespread distribution of this receptor among the classes of chordates, including deep-occurring teleosts (Siebenaller and Murray, 1986, 1988), and we had also identified potentially adaptive differences in ligand binding among species (Murray and Siebenaller, 1987; Siebenaller and Murray, 1988). Agonist occupation of the A₁ adenosine receptor inhibits cAMP accumulation in mammalian central nervous tissue preparations (reviews by Wolff *et al.*, 1981; Londos *et al.*, 1983; Snyder, 1985; Williams, 1987). The A₁ adenosine receptor is coupled to adenylyl cyclase [ATP pyrophosphatase (cyclizing); EC 4.6.1.1] by an inhibitory guanine nucleotide binding protein (G_i protein). Agonist occupation of the other subclass of adenosine receptor coupled to adenylyl cyclase, the A₂ receptor, stimulates adenylyl cyclase activity. These receptors are further distinguished on the basis of the rank order potencies of adenosine analogs (Daly, 1983a, b; Stone, 1985; Williams, 1987).

At a measurement temperature of 22°C, the binding affinities, specificities, and pharmacological profiles of the A₁ adenosine receptors in teleost fishes are similar to those of mammals (Siebenaller and Murray, 1986; Murray and Siebenaller, 1987). The binding of agonists to the high affinity state of mammalian A₁ receptors is disrupted by the low temperatures typical of body temperatures of many cold-adapted fishes (e.g., Bruns *et al.*, 1980; Trost and Schwabe, 1981; Murphy and Snyder, 1982; Lohse *et al.*, 1984; Siebenaller and Murray, 1988). However, agonist recognition and binding properties of the A₁ adenosine receptors are retained in evolutionary adaptation to different body temperatures. For instance,

for eight vertebrate species with body temperatures of 1–40°C, K_d values for the agonist N⁶-[³H]cyclohexyladenosine ([³H]CHA) measured at 5°C varied 30-fold; however, the binding affinities vary only four-fold when compared at temperatures similar to the species' body temperatures (Siebenaller and Murray, 1988).

Materials and Methods

Specimens

Demersal adult *Antimora rostrata* (Moridae) were collected by otter trawl at their depths of typical abundance (850–2500 m) off the coast of Newfoundland, Canada, on a cruise of the R/V *Gyre* in May 1986. Brain tissue was dissected, frozen in liquid nitrogen at sea, and transported to the laboratory where tissues were maintained at –80°C until used. For the [³²P]ADP-ribosylation experiments described below, brain tissue from the macrourids, *Macrourus berglax*, *Coryphaenoides rupestris*, and *C. armatus*, taken off the coast of Newfoundland, the scorpaenids, *Sebastolobus alascanus* and *S. altivelis*, taken on a cruise of the R/V *Vecoma* off the coast of Oregon, and the salmonid, *Oncorhynchus mykiss* (*Salmo gairdneri*), raised at the Food Toxicology and Nutrition Laboratory of Oregon State University, were also used. The macrourid species were chosen because they represent a primarily deep-sea family. The *Sebastolobus* species have been employed in a variety of pressure adaptation studies (Siebenaller and Somero, 1989), and *O. mykiss* is a pelagic freshwater species.

Reagents

[Adenylate-³²P]-nicotinamide adenine dinucleotide ([³²P]NAD, 31.31 Ci/mmol), [³H]CHA (34.4 Ci/mmol), [α-³²P]ATP (800 Ci/mmol) and [³H]cAMP (30.5 Ci/mol) were from DuPont NEN (Wilmington, Delaware). The R- and S-diastereomers of N⁶-phenylisopropyladenosine (PIA), 5'-N-ethylcarboxamidoadenosine (NECA), and papaverine were obtained from Research Biochemicals, Inc. (Wayland, Massachusetts). Pertussis toxin was from List Biological Laboratories (Campbell, California). Electrophoresis reagents and molecular weight standards were from Bio-Rad (Richmond, California). Adenosine deaminase (Sigma, Type VI), N⁶-cyclopentyladenosine (CPA), 2-chloroadenosine, and all other chemicals used were from Sigma Chemical Co. (St. Louis, Missouri). Water was processed through a four-bowl Milli-Q purification system (Millipore, Bedford, Massachusetts).

Preparation of brain membranes

Antimora rostrata brain membranes for assays of ligand binding were prepared following the procedures described by Murray and Siebenaller (1987).

For adenylyl cyclase assays, brain tissue was disrupted with a Dounce (pestle A) in 100 volumes of 10 mM HEPES, pH 7.6 at 5°C, and centrifuged at $27,000 \times g$ (0–4°C) for 10 min. The pellet was resuspended in buffer, centrifuged at $27,000 \times g$ for 10 min, resuspended in buffer, and 7.5 units/ml of adenosine deaminase were added. The homogenate was incubated at 18°C for 30 min, chilled on ice, centrifuged at $27,000 \times g$, and the pellet resuspended in buffer and 7.5 units/ml adenosine deaminase. Fifty microliters of this homogenate were used in the adenylyl cyclase assays.

For ADP-ribosylation experiments, membranes were homogenized with a Dounce (pestle A) in 40 volumes of 50 mM Tris-HCl, pH 7.6 at 5°C. The homogenate was centrifuged at $27,000 \times g$ for 10 min. The pellet was resuspended in 40 volumes of Tris-HCl buffer. Fifty microliters of this were used for the ribosylation experiments.

Protein was determined by the method of Lowry *et al.* (1951) following solubilization of the samples in 0.5 M NaOH. Bovine serum albumin (Sigma Chemical Co.) was used as the standard.

Time course of agonist association and dissociation

Aliquots of brain membranes were incubated with 2.85 nM [³H]CHA in 50 mM Tris-HCl, pH 7.6 at the incubation temperature of 5°C. Nonspecific binding was determined simultaneously in the presence of 60 μM R-PIA. For the dissociation experiments, samples were first incubated at 5°C with 2.85 nM [³H]CHA for 240 min to allow binding to reach equilibrium. R-PIA (60 μM) was added in a negligible volume (1% of the total) to initiate the dissociation reaction. Samples were started at timed intervals, and all incubations were terminated simultaneously by filtration over No. 32 glass fiber filter strips (Schleicher and Schuell Inc., Keene, New Hampshire) using a cell harvester (Brandel Instruments, Gaithersburg, Maryland). The data were analyzed as described below.

Equilibrium binding assay for membrane bound A₁ adenosine receptors

The rapid filtration assay described by Bruns *et al.* (1980) and Murray and Cheney (1982) was used with minor modifications to determine the specific binding of the A₁-selective agonist [³H]CHA to *A. rostrata* brain membranes. Assays were conducted in Tris-HCl, pH 7.6, at the incubation temperature of 5°C. The procedures described in Siebenaller and Murray (1988) were followed. Brain membrane protein (0.4–1.2 mg) was added to each assay tube.

[³²P]ADP-ribosylation

Pertussis toxin-catalyzed [³²P]ADP-ribosylation of GTP binding proteins followed the procedures described

in Ribeiro-Neto *et al.* (1985) and Greenberg *et al.* (1987). The 100-μl incubation mixture contained 100 mM Tris-HCl, pH 7.5, at the incubation temperature of 5°C, 25 mM dithiothreitol, 2 mM ATP, 0.1 mM GTP, 5 μCi [³²P]-NAD, 1.5 μg soybean trypsin inhibitor, 15 μg bacitracin, 2 μg pertussis toxin, and 37–92 μg membrane protein. After 3 h, the reaction was stopped by adding 50 μl of stop solution (3% sodium dodecyl sulfate, 42% glycerol, 15% 2-mercaptoethanol, 200 mM Tris-HCl, pH 6.8, at 20°C) and boiled for 5 min. The denatured samples were subjected to sodium dodecyl sulfate polyacrylamide electrophoresis in a 1.5-mm thick 12.5% acrylamide gel following Laemmli (1970). The gel was stained with 0.25% Serva Blue R (Serva Fine Biochemicals, Westbury, New York) in 25% 2-propanol, 10% acetic acid, destained and dried. The dried gels were exposed to Kodak (Rochester, New York) X-Omat AR film. DuPont Cronex Lightning Plus intensifying screens were used.

Adenylyl cyclase assays

The standard adenylyl cyclase assay contained in a total volume of 150 μl, 10 to 20 μg of *A. rostrata* brain membrane protein, 50 mM HEPES, pH 7.6 at the assay temperature of 5°C, 50 μM 2-deoxy-ATP, approximately 1 to 1.5×10^6 cpm [α -³²P]ATP, 10 μM GTP, 6.25 mM Mg acetate, 100 mM NaCl, 7.5 units creatine kinase, 5 mM phosphocreatine, 1.5 μg soybean trypsin inhibitor, 15 μg bacitracin, and other constituents as indicated below. Assays were conducted in triplicate in a refrigerated water bath for 2 h. The reaction was stopped by adding 250 μl of 2% sodium dodecyl sulfate, 45 mM ATP, and 1.3 mM cAMP. The samples were boiled for 3 min and 600 μl of water were added. [³²P]cAMP generated in the assays was determined according to Salomon *et al.* (1974).

For assays of the effects of hydrostatic pressure on adenylyl cyclase activity and inhibition, samples were transferred to polyethylene tubing. The tubing was trimmed to exclude air bubbles and sealed using a pipet heat sealer. [³H]cAMP (approximately 20,000 cpm) was used as an internal standard to monitor the recovery of sample through the sealing and incubation, and through the subsequent column chromatography steps isolating the [³²P]cAMP from the [³²P]ATP following Salomon *et al.* (1974). The pK_a of HEPES, the buffer used in these experiments, is relatively insensitive to pressure (Bernhardt *et al.*, 1988). Samples were incubated in high pressure vessels maintained at 5°C in a refrigerated circulating water bath. The high pressure apparatus is described in Hennessey and Siebenaller (1985). Samples were incubated for 120 min. The time required to seal and pressurize a group of four samples and the time required to re-

move the samples was less than 6% of the incubation time at elevated pressure. Samples sealed and incubated at atmospheric pressure have adenylyl cyclase activities identical to samples that are incubated in test tubes.

Data analysis

The kinetic parameters for the time course of association and dissociation of specific [^3H]CHA binding were estimated using the equations of Weiland and Molinoff (1981). Rate constants were calculated by least squares linear regression.

The association data were analyzed as a pseudo-first order reaction described by the equation:

$$\ln \frac{[B_{\text{eq}}]}{([B_{\text{eq}}] - [B])} = ([L]k_{+1} + k_{-1})t = k_{\text{obs}}t$$

where $[B_{\text{eq}}]$ is the amount of [^3H]CHA bound at equilibrium, $[B]$ is the amount bound at time t , $[L]$ is the concentration of [^3H]CHA. The pseudo-first order rate constant, k_{obs} is determined from the slope of plots of $\ln [B_{\text{eq}}]/([B_{\text{eq}}] - [B])$ versus time (Weiland and Molinoff, 1981; Kitabgi *et al.*, 1977).

The first order dissociation constant (k_{-1}) was determined from a plot of the equation:

$$\ln [B]/[B_0] = k_{-1}t$$

where $[B_0]$ is the concentration of bound [^3H]CHA at time 0. The ratio of the rate constants (k_{-1}/k_{+1}) provides an estimate of the equilibrium dissociation constant (K_d) for [^3H]CHA binding.

Saturation isotherms were analyzed using LUNDON-1 (Lundon Software, Inc., Cleveland, Ohio) iterative curve fitting routines (Lundeen and Gordon, 1985).

Concentration-response data for inhibition of adenylyl cyclase were analyzed by fitting a three parameter logistic equation to the data. The equation used was:

$$Y = \frac{E - I}{1 + \frac{X}{(IC_{50})}} + I$$

where Y is the rate of adenylyl cyclase activity ($\text{pmol cAMP min}^{-1} \text{mg protein}^{-1}$) in the presence of a given concentration of adenosine analog (X); E is the activity of adenylyl cyclase in the absence of adenosine analog; I is the activity in the presence of a maximally inhibiting concentration of adenosine analog; and IC_{50} is the concentration of adenosine analog that produces a half-maximal inhibition of the adenylyl cyclase activity. The concentration-response data were analyzed with an unweighted nonlinear regression analysis program using FITFUN, a computer modeling program on the PROPHET II Computer System.

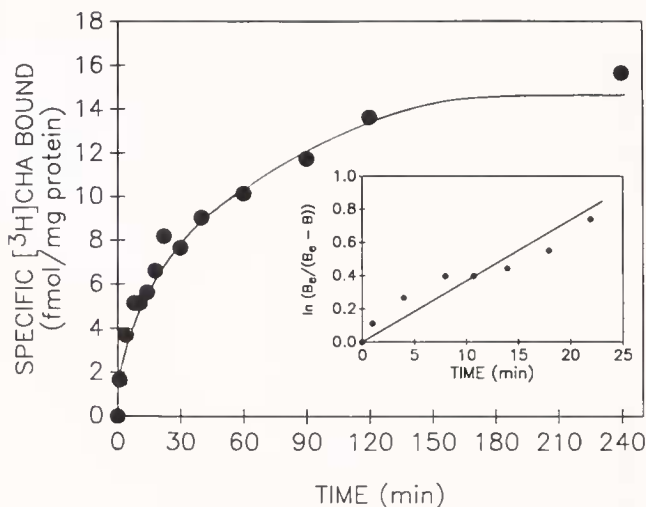


Figure 1. Time course of association of specific [^3H]CHA binding to *Antimora rostrata* brain membranes at 5°C. [^3H]CHA (2.85 nM) was incubated at 5°C with 0.52 mg membrane protein per tube prepared as described in Materials and Methods. Nonspecific binding was measured in the presence of 60 μM R-PIA and was constant throughout the association reaction. Inset shows the pseudo-first order replot of [^3H]CHA association data. A single representative experiment is shown.

Results

Time course of association and dissociation

At 5°C, the agonist [^3H]CHA bound specifically and reversibly to the *A. rostrata* brain membranes. By 120 min, the specific binding of [^3H]CHA was 87% of that observed at 240 min (Fig. 1). Nonspecific binding was constant throughout the association reaction. The k_{obs} calculated from the plot of $\ln [B_{\text{eq}}]/([B_{\text{eq}}] - [B])$ against time was $0.0484 \pm 0.00719 \text{ min}^{-1}$ (Fig. 1, inset). Examination of the data in the inset suggest that a multiple parameter fit might provide a better fit. However, we approximated k_{obs} by a single parameter because we did not have sufficient data to fit a multiple parameter model. The dissociation rate constant (k_{-1}) was determined directly by following the dissociation of specifically bound [^3H]CHA by the addition of 60 μM R-PIA. The [^3H]CHA was readily dissociated on addition of excess R-PIA (Fig. 2). The k_{-1} calculated from the plot of $\ln [B]/[B_0]$ versus time was $0.0136 \pm 0.0006 \text{ min}^{-1}$ (Fig. 2, inset). K_d of [^3H]CHA was estimated from the ratio of k_{-1}/k_{+1} . This kinetically derived estimate of the equilibrium dissociation constant, K_d , was 1.11 nM.

Equilibrium saturation analysis

The specific binding of [^3H]CHA at 5°C to *A. rostrata* brain membranes was saturable (Fig. 3). Specific binding, defined as total binding minus nonspecific binding

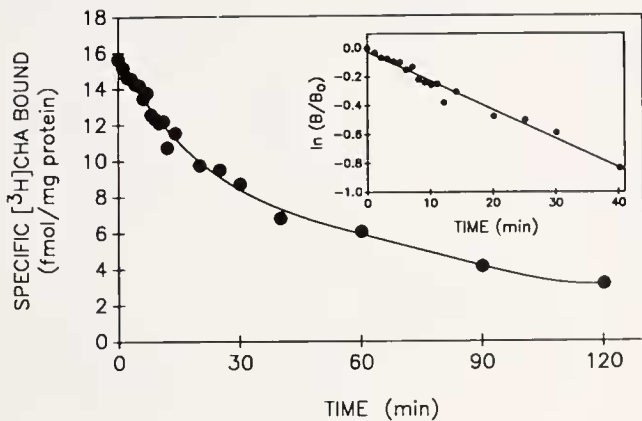


Figure 2. Time course of R-PIA-induced dissociation of specific [^3H]CHA binding in *Antimora rostrata* brain membranes. Membranes (0.52 mg protein) were first incubated at 5°C with $2.85\ \mu\text{M}$ [^3H]CHA for 240 min to allow binding to reach equilibrium. Sixty μM R-PIA was added in a negligible volume (1% of the total incubation volume) to initiate the dissociation reaction. The nonspecific binding, which has been subtracted from each experimental point, was determined in the presence of $60\ \mu\text{M}$ R-PIA. Inset depicts the first-order replot of dissociation data and represents the best least-squares regression line. Data shown are from a single experiment.

determined in the presence of $30\ \mu\text{M}$ R-PIA, saturated over the range of 0.096 to approximately $16\ \text{nM}$. Nonspecific binding increased linearly as a function of [^3H]CHA concentration. Analysis of the [^3H]CHA saturation isotherm, using equations based on mass action principles, indicated that the data were adequately described by interaction with a single high affinity state of the receptor. Computer modeling of replicate experiments

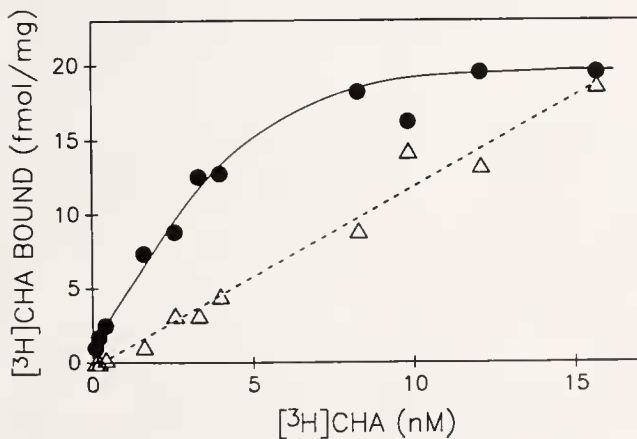


Figure 3. Equilibrium saturation binding of [^3H]CHA to *Antimora rostrata* brain membranes at 5°C . Membranes were incubated 150 min with 11 concentrations of free [^3H]CHA ranging from 0.096 to $15.7\ \text{nM}$ in this experiment. Specific binding is indicated by the filled circles. Nonspecific binding, shown by the open triangles, was determined in the presence of $30\ \mu\text{M}$ R-PIA.

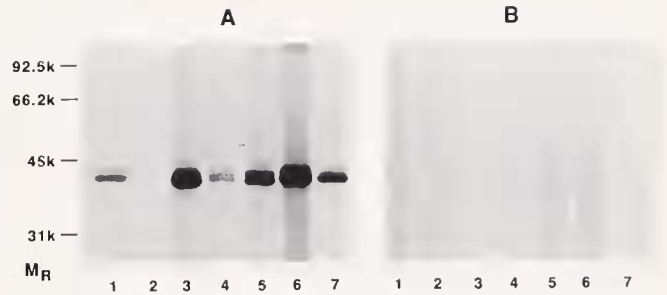


Figure 4. Autoradiogram of teleost fish brain membrane preparations [^{32}P]ADP-ribosylated by pertussis toxin at 5°C . Preparations were incubated for 2 h with [^{32}P]NAD with (A) or without (B) pertussis toxin. The samples were denatured and subjected to sodium dodecyl sulfate polyacrylamide electrophoresis in 12.5% acrylamide gels following Laemmli (1970). The gels were dried and exposed to x-ray film. 1. *Antimora rostrata*, 2. *Sebastolobus alascanus*, 3. *S. alivelis*, 4. *Macrurus berglax*, 5. *Coryphaenoides rupestris*, 6. *C. armatus*, 7. *Oncorhynchus mykiss*.

yielded an average K_d value of $4.86 \pm 0.95\ \text{nM}$ with a density of $25.6 \pm 2.02\ \text{fmol mg membrane protein}^{-1}$. The K_d value obtained from the saturation isotherm is in relatively good agreement with the kinetically derived K_d value of $1.11\ \text{nM}$.

[^{32}P]ADP-ribosylation

Figure 4 shows an autoradiogram of a sodium dodecyl sulfate Laemmli gel used to resolve brain membrane preparations incubated with [^{32}P]NAD in the presence and absence of activated pertussis toxin. [^{32}P]ADP-ribosylation did not occur in the absence of pertussis toxin. Substrates of approximately 39,000 to 41,000 Da apparent molecular mass were specifically labeled in the brain membrane preparations from each of the seven teleost species surveyed. In the autoradiogram some preparations clearly have two labeled substrates, e.g., *Macrurus berglax* and *Coryphaenoides rupestris* (Fig. 4). The ribosylation reaction was performed at 5°C to maintain membrane viscosity close to the body temperatures of these species. At this temperature, pertussis toxin-catalyzed ribosylation of membranes from cold-adapted deep- and shallow-living marine teleosts and the freshwater species, *O. mykiss*, results in labeling of components similar to those of warm-adapted species. The relative molecular masses of the components ribosylated are in the range corresponding to the molecular masses reported for the alpha subunits of the GTP-binding proteins G_i and G_o , an "other" G protein of unknown function, in other species (41,000 Da and 39,000 Da, respectively; Gilman, 1987; Pfeuffer and Helmreich, 1988).

Adenylyl cyclase

The time courses for basal and $0.1\ \mu\text{M}$ forskolin-stimulated adenylyl cyclase activity in *A. rostrata* brain mem-

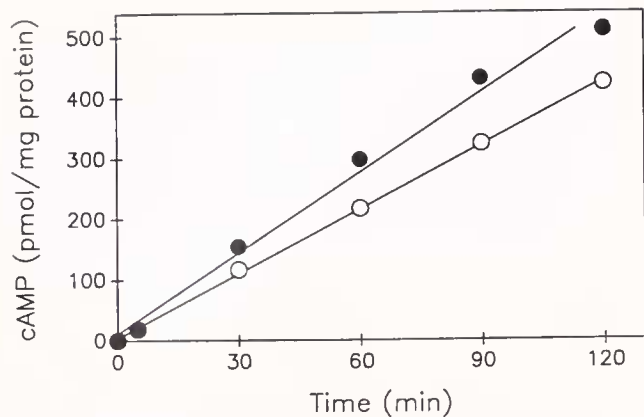


Figure 5. Time course of the adenylyl cyclase reaction at 5°C in brain membranes from *Antimora rostrata*. Open circles: basal adenylyl cyclase activity. Filled circles: forskolin (0.1 μ M) stimulated activity. Reaction mixture contained 50 mM HEPES, pH 7.6, at 5°C, 4 mM magnesium acetate, 50 μ M 2-deoxy ATP, 100 μ M cAMP, 200 μ M papaverine, 6.3 mg creatine phosphate, 2 mg creatine phosphokinase, 100 mM NaCl, 10 μ M GTP, and 2.5 units/ml adenosine deaminase.

branes are shown in Figure 5. At 5°C, both the basal and forskolin-stimulated rates were linear for at least 120 min. An incubation time of 120 min was used for all subsequent experiments.

The inhibition of basal adenylyl cyclase activity by N⁶-cyclopentyladenosine (CPA) was used as a biochemical measure of the extent of coupling of A₁ receptors to adenylyl cyclase via a guanine nucleotide regulatory protein. CPA is a highly selective A₁ adenosine receptor agonist and was used to eliminate potential interactions with the A₂ adenosine receptor (Bruns *et al.*, 1986; Williams *et al.*, 1986) or with the P-site on the catalytic subunit of adenylyl cyclase (Londos *et al.*, 1983; Blair *et al.*, 1989; Johnson *et al.*, 1989). The dependence of CPA-induced inhibition of adenylyl cyclase activity on GTP concentration is depicted in Figure 6. Basal adenylyl cyclase activity in the absence and presence of 100 μ M CPA is shown. CPA had little effect on adenylyl cyclase activity in the absence of added GTP; however, at GTP concentrations of 1 to 100 μ M, the A₁ selective agonist inhibited activity. A representative concentration response curve in Figure 7 depicts the inhibition of basal adenylyl cyclase activity in *A. rostrata* brain membranes by various concentrations of CPA. The IC₅₀ value for inhibition of adenylyl cyclase was $5.08 \pm 2.65 \mu$ M. The maximal inhibition of basal activity by CPA ranged from 7 to 17%. Other adenosine analog agonists, such as R-PIA, 2-chloroadenosine, and NECA displayed similar efficacies as inhibitors of adenylyl cyclase activity (data not shown). The concentration-dependent inhibition of adenylyl cyclase activity is consistent with the involvement of A₁ receptors in the inhibitory modulation of the enzyme.

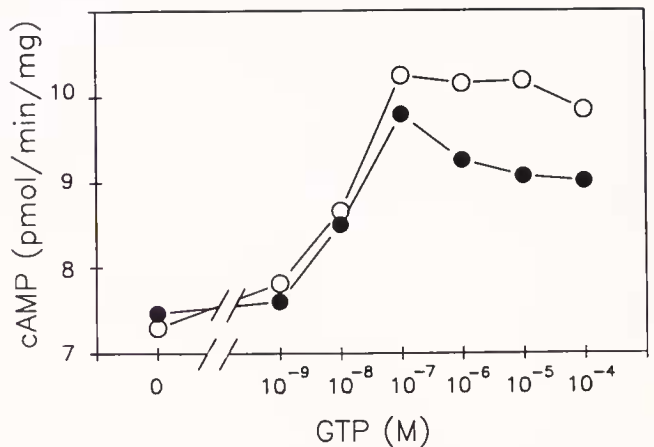


Figure 6. Effect of GTP concentration on *Antimora rostrata* brain membrane basal adenylyl cyclase activity in the absence (open circles) and presence (filled circles) of 100 μ M CPA at 5°C.

As shown in Figure 8, the basal adenylyl cyclase activity of the deep-living fish *A. rostrata* was unaffected by 272 atm of pressure, which is comparable to the pressures experienced by the species at the lower end of its depth range. At atmospheric pressure, basal adenylyl cyclase activity is $3.3 \pm 0.07 \text{ pmol min}^{-1} \text{ mg membrane protein}^{-1}$. Basal activity is unchanged at 272 atm ($3.3 \pm 0.11 \text{ pmol min}^{-1} \text{ mg}^{-1}$). The efficacy of the adenosine receptor agonists CPA (100 μ M) and NECA (100 μ M) as inhibitors of adenylyl cyclase were compared at atmospheric pressure and 272 atm. As shown in Figure 8, the increased pressure doubled the mean percentage inhibition of basal activity by NECA over that observed at 1 atm ($14 \pm 8\%$ at atmospheric pressure, $29 \pm 2\%$ at 272 atm) but had no effect on the inhibition by CPA ($9 \pm 5\%$ at atmospheric pressure and $8 \pm 5\%$ at 272 atm).

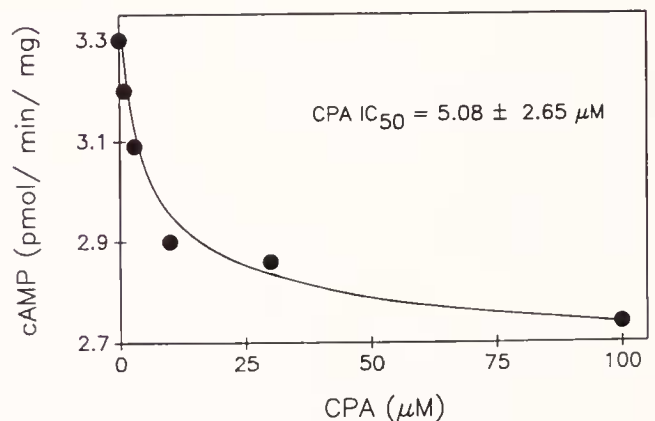


Figure 7. Inhibition of *Antimora rostrata* brain membrane basal adenylyl cyclase activity by the A₁ adenosine receptor agonist CPA at 5°C.

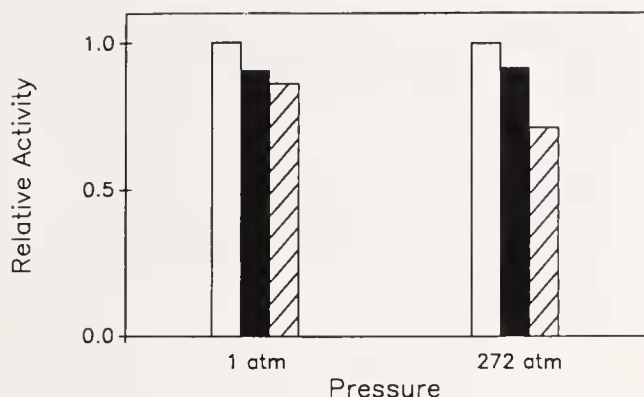


Figure 8. The effects of hydrostatic pressure on *Antimora rostrata* basal adenylyl cyclase activity (open bar) and inhibition of basal adenylyl cyclase activity by the adenosine analogs CPA (100 μ M; filled bar) and NECA (100 μ M; hatched bar). Membranes were incubated at atmospheric pressure or 272 atm pressure for 2 h at 5°C. All values are standardized to the 1 atm basal adenylyl cyclase activity. The 1 atm and 272 atm basal activities were 3.3 pmol min⁻¹ mg protein⁻¹. The 1 atm data are the mean of three replicates; the 272 atm values are the mean of six replicates. The average standard errors are 11.7% of the values of the mean.

Discussion

Binding of the agonist [³H]CHA to the A₁ receptor in *A. rostrata* brain membranes at 5°C is saturable and readily reversible (Figs. 1, 2, 3). At 5°C, the rate constants determined for *A. rostrata* from association-dissociation experiments are lower than those reported for two scorpaenid fishes, *Sebastolobus alascanus* and *S. altivelis*, at a measurement temperature of 22°C (Murray and Siebenaller, 1987). The k_{obs} for the *A. rostrata* binding reaction is only 21 to 25% of the values obtained for the *Sebastolobus* species at 22°C. The k_{-1} value for *A. rostrata* is only 40 to 65% of the 22°C values. At 22°C the binding reaction in *Sebastolobus* membranes is complete in 30 min. In contrast, at 5°C, the reaction in *A. rostrata* membranes takes more than four times as long to reach equilibrium (Fig. 1). At 5°C, which approximates the body temperatures of these three species, the K_d values are similar (Siebenaller and Murray, 1988).

At 5°C, the rank order potencies of agonists is compatible with that expected for the A₁ receptor (Fig. 9; Siebenaller and Murray, 1988). The K_i values indicate discrimination of the R- and S-diastereomers of PIA (4.5 and 115.9 nM, respectively). The rank order potency series expected for A₁ receptors is R-PIA \geq 2-chloroadenosine \geq NECA > S-PIA, and for A₂ receptors NECA > 2-chloroadenosine > R-PIA \geq S-PIA (Daly, 1983 a, b; Stone, 1985; Williams, 1987). The rank order potencies, the discrimination between R-PIA and S-PIA, and the K_d of [³H]CHA values are characteristic of an A₁ adenosine receptor.

The substrates specifically [³²P]ADP-ribosylated by pertussis toxin in *A. rostrata* and six other teleost species (Fig. 4) have apparent molecular masses characteristic of the class of alpha subunits from the guanine nucleotide binding regulatory proteins G_i and G_o (Gilman, 1987; Pfeuffer and Helmreich, 1988). The GTP-dependence of CPA-induced inhibition of cAMP accumulation (Fig. 6) demonstrates a role for these G proteins in the coupling of the A₁ receptor to negative modulation of adenylyl cyclase activity in *A. rostrata* brain membranes. In the presence of GTP, CPA inhibited basal adenylyl cyclase activity with an IC₅₀ of $5.08 \pm 2.65 \mu$ M (Fig. 7). The maximal inhibition ranged from 7 to 17%. This degree of inhibition is similar to the maximal inhibition of adenylyl cyclase by CPA in embryonic chick heart membranes (Blair *et al.*, 1989).

The A₁ adenosine receptor of the deep-living teleost, *Antimora rostrata*, is capable of modulating the activity of adenylyl cyclase under the conditions of low temperature and high hydrostatic pressure, which characterize the bathyal habitat (Fig. 8). Experiments currently underway, using brain tissues from other species, indicate that the A₁ adenosine receptor-G_i-adenylyl cyclase system can be markedly perturbed by hydrostatic pressures less than the 272 atm used in the present study. For instance, in shallower-occurring fishes, basal adenylyl cyclase activity is inhibited 11 to 25% by 136 atm pressure (Siebenaller and Murray, work in progress). In contrast, *A. rostrata* brain tissue adenylyl cyclase is unaffected by 272 atm pressure, the highest pressure tested. The efficacy of agonists at the A₁ adenosine receptor is not lessened by increased pressure (Fig. 8). CPA-induced inhibition of adenylyl cyclase was unaltered, and the efficacy of NECA increased. Thus, basal adenylyl cyclase activity, as well as signal transduction by the A₁ receptor sys-

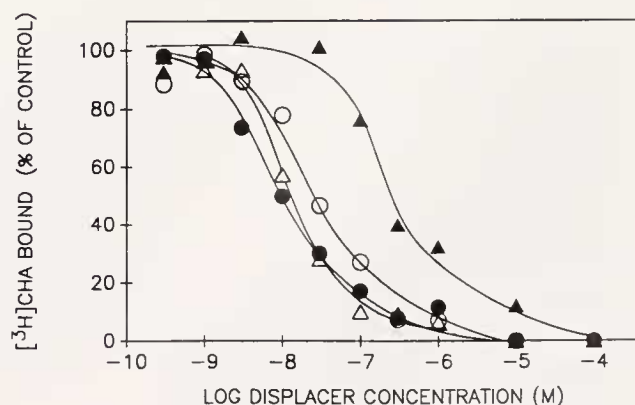


Figure 9. Inhibition of specific [³H]CHA binding in *Antimora rostrata* brain membranes by adenosine analogs: R-PIA (open circle), NECA (open triangle), 2-chloroadenosine (filled circle), S-PIA (filled triangle). Eleven concentrations of each analog were incubated with membranes and 7.9 nM [³H]CHA for 150 min at 5°C.

tem, are functional under the conditions of pressure and temperature at which *A. rostrata* occurs.

Consideration of the effects of low temperature and high hydrostatic pressure on membrane viscosity (Cossins and Macdonald, 1989) suggests that any of the components of the A_1 receptor- G_i protein-adenylyl cyclase complex may be susceptible to perturbation in organisms colonizing the deep sea. For *A. rostrata*, the function of this transmembrane signaling complex is maintained at low temperature and high hydrostatic pressure. The pressure insensitivity of this membrane-associated system in *A. rostrata* is analogous to the pressure adaptations observed for cytoplasmic proteins (Siebenaller and Somero, 1989). The K_m values of NAD-dependent dehydrogenases of deep-living species are relatively insensitive to perturbation by pressure. In contrast, homologous enzymes from shallow-living, cold-adapted species are perturbed by pressures as low as 68 atm. By having pressure-resistant enzymes, function is preserved over the range of depths that may be experienced by an individual during ontogeny or diel vertical migrations, or by a species maintaining populations over a broad depth gradient (Siebenaller, 1987).

Gibbs and Somero (1989) hypothesized, based on their study of Na^+/K^+ -ATPase in teleost gill tissue, that clear adaptations of membrane-associated systems to pressure may only be apparent in species occurring at depths greater than 2000 m. Although our data do not directly test this hypothesis, the pressure insensitivity of the A_1 receptor and effector system in *A. rostrata*, which commonly occurs to depths of 2500 m, are compatible with their suggestion. This pressure resistance in *A. rostrata* is a standard with which to compare the effects of environmental parameters on transmembrane signal transduction in other deep- and shallow-occurring species.

Acknowledgments

This research was supported by NSF grant DCB-8710155, and ONR contracts N00014-88-K-0426, N00014-88-K-0432, and ONR grants N00014-89-J-1865 and N00014-89-J-1869. Shiptime on the R/V *Wecoma* off the coast of Oregon was supported by NSF grant DCB-8710155. Shiptime on the R/V *Gyre* off the coast of Newfoundland was supported by NSF grant DMB-8502857 to Dr. A. F. Riggs. We thank Drs. A. Riggs and R. Noble for their help in obtaining specimens and Drs. P. Franklin and M. Leid for their help with the ADP-ribosylation experiments.

Literature Cited

- Avrova, N. F. 1984. The effect of natural adaptations of fishes to environmental temperature of brain ganglioside fatty acid and long chain base compositions. *Comp. Biochem. Physiol.* **78B**: 903-909.
- Bernhardt, G., A. Distèche, R. Jaenicke, B. Koch, H.-D. Ludemann, and K.-O. Stetter. 1988. Effect of carbon dioxide and hydrostatic pressure on the pH of culture media and the growth of methanogens at elevated temperature. *Appl. Microbiol. Biotechnol.* **28**: 176-181.
- Blair, T. A., M. Parenti, and T. F. Murray. 1989. Development of pharmacological sensitivity to adenosine analogs in embryonic chick heart: role of A_1 adenosine receptors and adenylyl cyclase inhibition. *Mol. Pharmacol.* **35**: 661-670.
- Bruns, R. F., J. W. Daly, and S. H. Snyder. 1980. Adenosine receptors in brain membranes: binding of N^6 -cyclohexyl[3H]adenosine and 1,3-diethyl-8-[3H]phenylxanthine. *Proc. Natl. Acad. Sci. USA* **77**: 5547-5551.
- Bruns, R. F., G. H. Lu, and T. A. Pugsley. 1986. Characterization of the A_2 adenosine receptor labeled by [3H]NECA in rat striatal membranes. *Mol. Pharmacol.* **29**: 331-346.
- Chong, P. L.-G., and A. R. Cossins. 1983. A differential polarized phase fluorometric study of the effects of high hydrostatic pressure upon the fluidity of cellular membranes. *Biochemistry* **22**: 409-415.
- Cossins, A. R., and A. G. Macdonald. 1984. Homeoviscous theory under pressure. II. The molecular order of membranes from deep-sea fish. *Biochim. Biophys. Acta* **776**: 144-150.
- Cossins, A. R., and A. G. Macdonald. 1986. Homeoviscous adaptation under pressure. III. The fatty acid composition of liver mitochondrial phospholipids of deep-sea fish. *Biochim. Biophys. Acta* **860**: 325-335.
- Cossins, A. R., and A. G. Macdonald. 1989. The adaptations of biological membranes to temperature and pressure: fish from the deep and cold. *J. Bioenergetics Biomembranes* **21**: 115-135.
- Daly, J. W. 1983a. Adenosine receptors: characterization with radioactive ligands. Pp. 59-69 in *Physiology and Pharmacology of Adenosine Derivatives*, J. W. Daly, Y. Kuroda, J. W. Phillis, H. Shimizu, and M. Ui, eds. Raven, New York.
- Daly, J. W. 1983b. Role of ATP and adenosine receptors in physiological processes: summary and prospectus. Pp. 275-290 in *Physiology and Pharmacology of Adenosine Derivatives*, J. W. Daly, Y. Kuroda, J. W. Phillis, H. Shimizu, and M. Ui, eds. Raven, New York.
- DeLong, E. F., and A. A. Yayanos. 1985. Adaptation of the membrane lipids of a deep-sea bacterium to changes in hydrostatic pressure. *Science* **228**: 1101-1103.
- DeLong, E. F., and A. A. Yayanos. 1987. Properties of the glucose transport system in some deep-sea bacteria. *Appl. Environ. Microbiol.* **53**: 527-532.
- Gibbs, A., and G. N. Somero. 1989. Pressure adaptation of Na^+/K^+ -ATPase in gills of marine teleosts. *J. Exp. Biol.* **143**: 475-492.
- Gilman, A. G. 1987. G proteins: transducers of receptor-generated signals. *Ann. Rev. Biochem.* **56**: 615-649.
- Greenberg, A. S., S. I. Taylor, and C. Londos. 1987. Presence of a functional inhibitory GTP-binding regulatory component, G_i , linked to adenylyl cyclase in adipocytes of ob/ob mice. *J. Biol. Chem.* **262**: 4564-4568.
- Hennessey, J. P., Jr., and J. F. Siebenaller. 1985. Pressure inactivation of tetrameric lactate dehydrogenase homologues of confamilial deep-living fishes. *J. Comp. Physiol. B* **155**: 647-652.
- Hennessey, J. P., Jr., and J. F. Siebenaller. 1987. Pressure-adaptive differences in proteolytic inactivation of M_4 -lactate dehydrogenase homologues from marine fishes. *J. Exp. Zool.* **241**: 9-15.
- Hochachka, P. W., and G. N. Somero. 1984. *Biochemical Adaptation*. Princeton University Press, Princeton, NJ.
- Hochachka, P. W., ed. 1975. Pressure effects on biochemical systems of abyssal and midwater organisms: the 1973 Kona Expedition of the Alpha Helix. *Comp. Biochem. Physiol.* **52B**: 1-199.
- Iwamoto, T. 1975. The abyssal fish *Antimora rostrata* (Günther). *Comp. Biochem. Physiol.* **52B**: 7-11.

- Johnson, R. A., S.-M. H. Yeung, D. Stübner, M. Bushfield, and I. Shoshani. 1989. Cation and structural requirements for P site-mediated inhibition of adenylyl cyclase. *Mol. Pharmacol.* **35**: 681-688.
- Kitabgi, P., R. Carraway, J. Van Rietschoten, C. Granier, J. L. Morgat, A. Menez, S. Leeman, and P. Freychet. 1977. Neurotensin: specific binding to synaptic membranes from rat brain. *Proc. Natl Acad. Sci. USA* **74**: 1846-1850.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680-685.
- Lohse, M. J., U. Lenschow, and U. Schwabe. 1984. Two affinity states of R₁ adenosine receptors in brain membranes. Analysis of guanine nucleotide and temperature effects on radioligand binding. *Mol. Pharmacol.* **26**: 1-9.
- Londos, C., J. Wolff, and D. M. F. Cooper. 1983. Adenosine receptors and adenylyl cyclase interactions. Pp. 17-32 in *Regulatory Function of Adenosine*, R. M. Berne, T. W. Rall, and R. Rubio, eds. Martinus Nijhoff Publishers, The Hague, Boston, London.
- Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**: 265-275.
- Lundeen, J. E., and J. H. Gordon. 1985. Computer analysis of binding data. Pp. 31-49 in *Receptor Binding in Drug Research*, B. O'Brien, ed. Marcel Dekker, New York.
- Murphy, K. M. M., and S. H. Snyder. 1982. Heterogeneity of adenosine A₁ receptor binding in tissue. *Mol. Pharmacol.* **22**: 250-257.
- Murray, T. F., and D. L. Cheney. 1982. Localization of N⁶-cyclohexyl[³H]adenosine binding sites in rat and guinea pig brain. *Neuropharmacology* **21**: 575-580.
- Murray, T. F., and J. F. Siebenaller. 1987. Comparison of the binding properties of A₁ adenosine receptors in brain membranes of two congeneric marine fishes living at different depths. *J. Comp. Physiol. B* **157**: 267-277.
- Pfeuffer, T., and E. J. M. Helmreich. 1988. Structural and functional relationships of guanosine triphosphate binding proteins. *Current Topics in Cellular Regulation* **29**: 129-216.
- Ribeiro-Neto, F. A. P., R. Mattera, J. D. Hildebrandt, J. Codina, J. B. Field, L. Birnbaumer, and R. D. Sekura. 1985. ADP-ribosylation of membrane components by pertussis and cholera toxin. *Methods Enzymol.* **109**: 566-572.
- Salomon, Y., C. Londos, and M. Rodbell. 1974. A highly sensitive adenylyl cyclase assay. *Analyt. Biochem.* **58**: 541-548.
- Siebenaller, J. F. 1987. Biochemical adaptation in deep-sea animals. Pp. 33-48 in *Current Perspectives in High Pressure Biology*, H. W. Jannasch, A. M. Zimmerman, and R. E. Marquis, ed. Academic Press, London.
- Siebenaller, J. F., and T. F. Murray. 1986. Phylogenetic distribution of [³H]cyclohexyladenosine binding sites in nervous tissue. *Biochem. Biophys. Res. Commun.* **137**: 182-189.
- Siebenaller, J. F., and T. F. Murray. 1988. Evolutionary temperature adaptation of agonist binding to the A₁ adenosine receptor. *Biol. Bull.* **175**: 410-416.
- Siebenaller, J. F., and G. N. Somero. 1978. Pressure-adaptive differences in lactate dehydrogenases of congeneric fishes living at different depths. *Science* **201**: 255-257.
- Siebenaller, J. F., and G. N. Somero. 1979. Pressure-adaptive differences in the binding and catalytic properties of muscle-type (M₄) lactate dehydrogenase of shallow- and deep-living marine fishes. *J. Comp. Phys.* **129**: 295-300.
- Siebenaller, J. F., and G. N. Somero. 1989. Biochemical adaptation to the deep sea. *Rev. Aquatic Sci.* **1**: 1-25.
- Small, G. J. 1981. A review of the bathyal fish genus *Antimora* (Moridae: Gadiformes). *Proc. California Academy Sci.* **42**: 341-348.
- Snyder, S. H. 1985. Adenosine as a neuromodulator. *Ann. Rev. Neurosci.* **8**: 103-124.
- Somero, G. N., and J. F. Siebenaller. 1979. Inefficient lactate dehydrogenases of deep-sea fishes. *Nature* **282**: 100-102.
- Stone, T. W. 1985. Summary of a symposium on purine receptor nomenclature. Pp. 1-5 in *Purines: Pharmacology and Physiological Roles*, T. W. Stone, ed. UCH Publishers, Deerfield Beach, Florida.
- Trost, T., and U. Schwabe. 1981. Adenosine receptors in fat cells. Identification by (-)-N⁶-[³H]phenylisopropyladenosine binding. *Mol. Pharmacol.* **19**: 228-235.
- Weiland, G. A., and P. B. Molinoff. 1981. Quantitative analysis of drug-receptor interactions: I. Determination of kinetic and equilibrium properties. *Life Sci.* **29**: 313-330.
- Wenner, C. A., and J. A. Musick. 1977. Biology of the morid fish, *Antimora rostrata*, in the western North Atlantic. *J. Fish. Res. Board Can.* **34**: 2362-2368.
- Williams, M. 1987. Purine receptors in mammalian tissues: pharmacology and functional significance. *Ann. Rev. Pharmacol. Toxicol.* **27**: 315-345.
- Williams, M., A. Braunwalder, and T. E. Erickson. 1986. Evaluation of the binding of the A-1 selective adenosine radioligand, cyclopentyladenosine (CPA), to rat brain tissue. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **332**: 179-183.
- Wolff, J., C. Londos, and D. M. F. Cooper. 1981. Adenosine receptors and the regulation of adenylyl cyclase. *Adv. Cyclic Nucleotide Res.* **14**: 199-214.
- Yancey, P. H., and J. F. Siebenaller. 1987. Coenzyme binding ability of homologs of M₄ lactate dehydrogenases. *Biochim. Biophys. Acta* **924**: 483-491.